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14. ABSTRACT Fe65 is a protein with established functions in neuronal cells. Our studies in the research period have shown that Fe65 is expressed in breast epithelial cells and that the expression is increased in breast cancers (BCa) and tumor tissue samples. It acts as an estrogen receptor alpha (ERα) transcriptional coregulator recruited by 17β-estradiuol to the promoters of estrogen target genes. Deletion analyses mapped the ERα binding domain to the phosphotyrosine binding domain 2. Ectopic Fe65 increased the transcriptional activity of the ERα in a PTB2 dependent manner in reporter assays. Fe65 knockdown decreased and its stable expression increased the activity of endogenous ERα in BCa cells and estrogen-induced target gene expression, ERα and coactivators recruitments to target gene promoters as well as BCa cell growth. Furthermore, Fe65 expression decreased the antagonistic activity of tamoxifen, suggesting a potential role for Fe65 in tamoxifen resistance. While a role of Fe65 in DNA damage induced p53 and γH2A.x phosphorylation was detected, Fe65 did not exhibit an effect on the overall ATM activity, suggesting that Fe65 may regulate DNA damage response by altering the interaction between ATM and its substrates. The studies are the first to define an important yet complex role for the Fe65 neuronal adaptor in BCa cells and have fully supported our original hypothesis that Fe65 activates the ERα to stimulate gene expression and BCa cell growth.

15. SUBJECT TERMS

Fe65, Breast Cancer, Estrogen Receptor, Tip60, Transgenic Mice

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INTRODUCTION

Fe65 is a multidomain adaptor protein with established functions in neuronal cells and neuro-degeneration diseases [1]. It forms a multimeric complex with Aβ amyloid precursor protein (APP) and histone acetyl transferase Tip60 to regulate the expression of genes [2]. Our published studies showed that Fe65 formed a complex with estrogen receptor α (ERα), which permits estrogen (E2) protection of neuronal cell from apoptosis induced by the APP-Tip60 transcriptional complex [3]. Unpublished studies produced preliminary evidences for the expression of a Fe65 isoform in breast epithelial cells as well as evidences for its overexpression in human breast tumors. Although Tip60 is a tumor suppressor that sensitizes cellular response to DNA damage [4, 5], Fe65-null MEFs were found to be more sensitive to DNA damage induced cell death [6], suggesting that Fe65 may oppose Tip60 activity in DNA repair. Furthermore, estrogens (E2) were shown to protect MCF-7 breast cancer (BCa) cells from DNA damage-induced apoptosis [7]. Based on the above information, we proposed a novel function for Fe65 in breast cancer cells. We hypothesize *that* ***Fe65 functions as a dual adaptor for ERα and Tip60 in breast cells to promote breast tumorigenesis. By bringing Tip60 to ERα target gene promoters, Fe65 enhances the mitogenic response of BCa cells to E2 and diminish the anti-estrogenic effect of tamoxifen (TAM). In addition, by bringing ERα to Tip60, Fe65 permits the tumor suppressive effect of Tip60, including its role DNA damage response as well as its transcriptional activity, to be suppressed by E2 and enhanced by TAM.*** To test this hypothesis, the funded grant was to achieve three specific aims. In Aim 1, protein interaction studies will be performed to define Fe65 domains that mediate its interaction with ERα and determine whether Fe65 knockdown decreases the growth response of BCa cells to E2 and enhances the growth suppressive effect of TAM through the recruitment of coactivators like Tip60 to estrogen target genes. In Aim 2, DNA damage repair assays will be performed to determine whether the tumor suppressor activity of Tip60 is inhibited by E2 and activated by TAM and whether the effect of E2 and TAM on Tip60 depends on Fe65. In Aim 3: it was proposed to establish transgenic mouse lines in which Fe65 expression is under the control of MMTV promoter to test the oncogenic potential of Fe65 in mammary tumorigenesis by breeding with MMTV-Myc mice.

BODY

The studies in the entire funding period supported the concept that Fe65 is a positive estrogen receptor regulator in breast cancer cells as has been proposed in Aim #1. However, the studies about the role in Fe65 in DNA damage response generated data that support a mechanism different from what was proposed in Aim #2. In addition, the effort to generate transgenic Fe65 mice was met with technical difficulties. Some of the tasks in the original Statements of Work (SOW) were modified and a revised SOW for Year #3 was submitted in the report for Year #2 and approved. This final report will describe experimental effort according to the tasks described in the original SOW as well as the revised SOW for Year #3.

Task 1: **To generate stable MCF-7 and T47D stable clones in which Fe65 expression is depleted by RNAi mediated silencing**. This studies in this task were successfully completed. Instead of stable knockdown with shRNA vector, Fe65 knockdown was achieved with transient siRNA in T47D, BT474 and ZR75-1 cells. For the reason that FE65 is expressed at low levels in MCF-7 cells, we established stable Fe65 over expression clones in MCF-7 to support the data with Fe65 knockdown. The transient knockdown and stable clones were used successfully for subsequent studies to address the role of Fe65 in ERα actions. Some of the data were described in the reports for year #2 and #3. More mature data are presented in the attached manuscript (see Figs. 5-8)

Task 2: **To analyze the recruitment of ERα, Fe65, Tip60 and SRC1 to estrogen targt genes IGF1, Myc, Cathepsin D and EBAG9**. The studies in this task were successfully completed. Analyses with AIB1 (SRC3) were performed in the place of SRC1 because AIB1 was more relevant for ERα actions in breast cancer cells. The studies showed that Fe65 was recruited to the promoters of all 4 ERα target genes. More importantly, the chromatin immunoprecipitation (ChIP) assays showed that Fe65 is an important determinant for the promoter recruitment of ERα and its coactivation AIB1. Some of the data were described in the report for year #3. More mature data were presented in the attached manuscript as Figs. 6-8.

Task 3: **To determine the regulation of estrogen target genes by Western blot and real time RT-PCR.** The studies in this task were completed. The analyses showed that the Fe65 knockdown decreased and its stable over expression decreased the estrogen induction of C-Myc and cyclin D1 expression in breast cancer cells. Some of the data were described in the reports for year #2 and #3. More mature data are presented in the attached manuscript as Fig. 5,

Task 4: **To determine the grwoth resposne of the MCF-7 cell clones to E2 and TAM.** The studies in this task were completed. The analyses showed that the Fe65 knockdown decreased and its stable over expression increases the estrogen stimulation of breast cancer growth and that the Fe65 stable expression suppressed the antagonistic activity of tamoxifen in the inhibition of estrogen induced cells growth. Some of the data were described in the reports for year #2 and #3. More mature data are presented in the attached manuscript as Fig. 8.

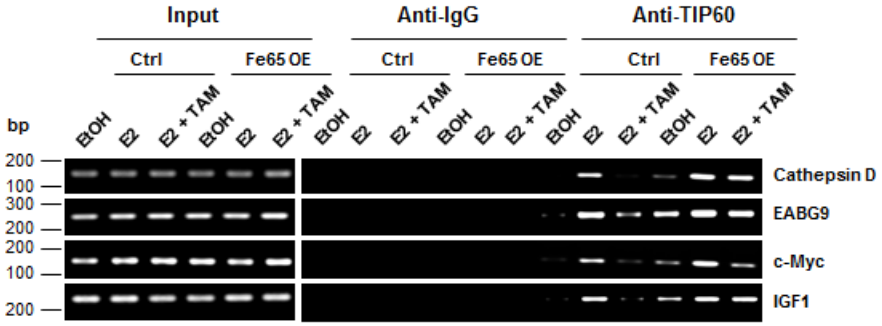

Fig 1. Tip60 was recruited to the promoters of ERα target genes and its recruitment is increased by Fe65 over expression. MCF-7 control (Ctrl) and Fe65 stably overexpressing (Fe65 OE) cells were estrogen starved for 72 hours and treated with vehicle (ethanol, EtOH), 10 nM 17β-estradiol (E2) or 10 nM E2 together with 1 μM 4-hydroxytamoxifen (TAM) for 45 minutes. Cells were processed for ChIP assays using control IgG or anti-Tip60 antibody. PCR were performed using primers specific for promoter regions of the 4 estrogen target genes as indicated. The numbers labeled on the left panel represent the DNA ladder, **bp**: base pairs.

Tasks 5, 6 and 8. **Studies were originally proposed to knock down Tip60 and to define the ATM activation, H2A.X phosphorylation and apoptosis in response to DNA damage in Fe65 knock down cells**. We had experienced difficulties in detecting Tip60 expression in Western blot with commercial antibodies. The exact reason is unclear but could be due to either poor Tip60 antibody for Western blot or the low Tip60 expression. Tip60 was shown to be a tumor suppressor and its expression is known to be decreased in breast cancers. The knockdown approach was abandoned.

In report for year #2**,** we described data showing that Fe65 knockdown decreased UV induced H2A.X phosphorylation and cell death as measured by PARP1 and caspase-3 cleavage. The data support a positive role of Fe65 in DNA damage response instead of a negative role as proposed initially. Interestingly, Fe65 expression was decreased by UV in ERα-negative cells and the decrease was protected by ectopic ERα, suggesting that ERα exert a suppressive role in DNA damage induced cell death by opposing the positive effect of Fe65. Consistently, we also showed that more ERα-positive cells survived UV treatments than ERα negative cells.

In ChIP assays, Tip60 was shown to be recruited to ERα target gene promoters and the recruitment was increased by Fe65 stable over expression in MCF-7 cells (Fig 1). However, when Tip60 was ectopically expressed, it did not potentiate the

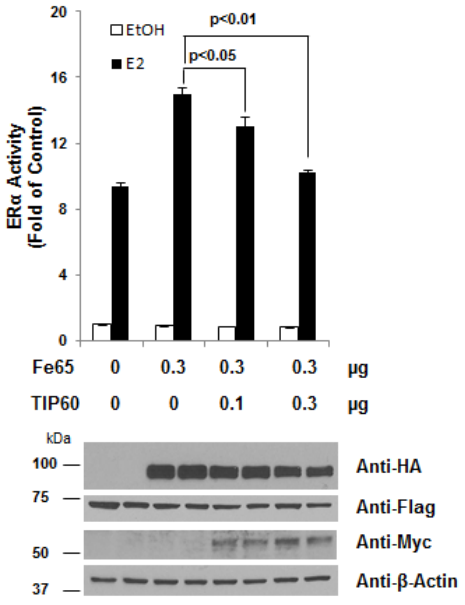

Fig 2. Tip60 did not potentiate the positive effect of Fe65 on the transcriptional activity of the ERα. Hela cells were plated in 12-well plates and transfected with 0.1 μg EREe1bluc, 0.1 μg pLENβGal and 0.2 μg Flag-ERα, together with indicated amounts of HA-Fe65 and Myc-Tip60. Cells were treated with vehicle (EtOH) or 10^{-8} M 17β-estradiol (E2) for 48 h. Luciferase activity was determined and normalized with β-gal activity. Folds of ERα activity was calculated by dividing the normalized luciferase activity with the activity of control cells transfected with empty vector and treated with EtOH. Western blot analyses were performed in parallel with lysates from transfected cells with indicated antibodies. β-actin blot was included to show even loading.

ERα activation induced by Fe65 (Fig 2), suggesting that Tip60 may not be involved in the functional interaction between Fe65 and ERα. Tip60 has been shown to be a tumor suppressor of which the expression, particularly the expression in the nucleus, is decreased on breast tumors [8]. Interestingly, it has been shown that estrogen-induced c-Myc and cyclin D1 mRNA expression were not affected by Tip60 depletion in MCF-7 cells [9] even though several non-growth related ERα target genes were altered, suggesting a selective involvement of Tip60 in ERα action in breast cancer cells. Although more studies are needed, it appears that estrogen regulation of breast cancer growth may not require Tip60.

Task. 7 and 9: **Studies initially proposed in tasks 7 and 9 in the funded project were to analyze Tip60 HAT activity and the recrruityment of Fe65 and ERα to the promoter of Tip60 targetr gene KAI1.** For the technical difficulties in detecting Tip60 and scientific concerns that estrogen regulation of breast cancer growth may not require Tip60 as described above, we replaced the studies initially proposed as tasks 7 and 9 with studies about p53 phosphorylation. The modification was submitted in Year#2 report and was approved in the revised SOW for Year #3.

In report for Year #3, we report that in two separate experiments, ATM activation by either UV or etoposide was not altered by Fe65 knockdown in MB-MDA-231 breast cancer cells. The data suggest that the earlier findings that Fe65 knockdown decreased H2A.X phosphorylation is not due to a reduction in overall ATM activity. The underlying mechanisms may be complex and may involve the changes in binding affinity between ATM and its substrates (such as H2A.X) or the changes of alternate kinases such as ATR and JNK, which are also known to phosphorylate H2A.X.

Besides H2A.X, p53 is another substrate for ATM kinase, of which the phosphorylation at Ser-15 is known to play an important role in DNA damage induced cell death. In the report for year #3, we presented data showing that p53 phosphorylation at Ser-15 in MDA-MB-231 cells was induced by both UV and etoposide treatments and Fe65 knockdown decreased p53 phosphorylation at Ser-15 by UV and etoposide.

The lack of a Fe65 effect on ATM activation by UV and etoposide suggest that the effect of Fe65 siRNA on p53 and H2A.X phosphorylation may be due to other kinases. In the literature, several other kinases are known to phosphorylate H2A.X in response to DNA damages, which include ATR, DNA-PK and JNK1 etc. In particular, JNK1 is also known to phosphorylate p53 and a recent paper showed that Fe65 in 293 cells was recruited to H2A.X which in turn recruited JNK1 to stimulate DNA damage induced cell death [9].

Task 10: ***To construct MMTV-Fe65 target gene and generated MMTV-Fe65 transgenic lines.*** The studies proposed in this task were completed. Based on genotyping and ex vivo imaging, more than eleven Fe65 transgenic lines were produced with the MMTV-HA-Fe65-IRES-Luc transgenic vectors. However, none of the lines expressed Fe65 in mammary gland. The effort and data were presented in the report for Years #1 and #2.

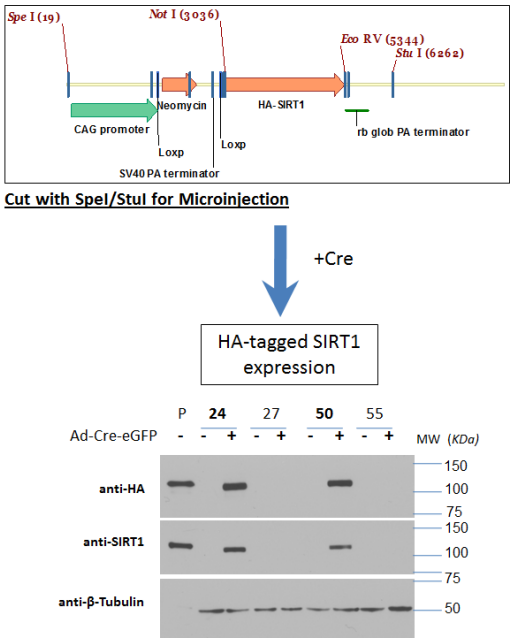

Fig 3. The Cre-inducible SIRT1 transgenic vector is shown at the top. The Western blot data are shown at the bottom. For the Western blot**,** mouse fibroblasts were isolated and pated onto 60 mm dishes. The cells were infected with 3 ul Ad-Cre-eGFP purchased from Baylor College of Medicine. 48 hours later, cells were lysed and celluklar extracts were subjected to Western blot with anti-HA mAb, MMS-101P, Covance, 1:2000) or Anti-SIRT1(rabbit, D739, CST, 1:1000). β-actin blot was included to show even loading.

Tasks 11 and 12: ***Studies initially proposed in Tasks 11 and 12 were to breed MMTV-Fe65 mice and MMTV-Myc and to monitor tumor formation in female mice of MMTV-Myc, MMTV-Fe65 and MMTV-Myc/Fe65.*** Due to the lack of Fe65 expression in mammary epithelium of the transgenic lines, we proposed to drop studies proposed in original tasks 11 and 12. Instead, we proposed to use SIRT1 gene as a marker gene to

validate the transgenic approaches we are using in Moffitt Cancer Center core facility to trouble-shoot why 11 lines were derived based on genotyping but the successful breeding did not yield any lines that express Fe65. The proposed modifications were approved in the revised SOW for Year #3.

We have generated new transgenic vectors for SIRT1 in which SIRT1 expression is inducible by the expression of Cre recombinase (Fig. 3, Top panel). The vectors were microinjected into mouse occytes and multiple founders were produced. Four transgenic lines were produced with stable transgenic DNA detected in F2 mice by genotyping, of which the inducible SIRT1 expression in primary mouse fibroblasts was detected in two (#24 and #50) (Fig 3, lower panel).

The studies suggest that the problems associated with earlier studies have something to do with either the MMTV promoter or due to the inclusion of IRES and Luciferase elements in the vector design, which make the vector very big in size. Alternatively, the failed Fe65 expression may be due to the toxicity of Fe65 in embryogenesis. New transgenic Fe65 vectors that is inducible by Cre has been constructed, which can be used in future studies to define the role of Fe65 mammary tumorigenesis *in vivo*.

KEY RESEARCH ACCOMPLISHMENTS

1. We have demonstrated for the first time that Fe65, a neuronal protein with a role in Alzheimer's disease, is widely expressed in breast epithelial cells and is over expressed in breast cancer cells and tumor tissues;

2. We have established Fe65 as an important positive regulator for ERα in breast cancer cells and defined it as a molecule that increases the recruitment of ERα and its coactivators to target gene promoters, a function that is uniquely different from typical ERα coactivators.

3. We have established a positive role for Fe65 in estrogen stimulation of breast cancer growth, projecting Fe65 as a potential molecular target to interfere with estrogen signaling and inhibit breast cancer growth.

4. We have also implicated Fe65 as a molecule that increases breast cancer's resistance to tamoxifen, projecting it as a potential molecular target to increase the clinical efficacy of tamoxifen, which is widely used for prevention and treatment of ER-positive breast cancers.

5. We have defined the interface for the Fe65-ERα interaction, providing a foundation for future structural analyses and drug development to disrupt the interaction and perturb estrogen actions for the purpose of breast cancer intervention.

6. Although the exact mechanisms remain to be defined, our studies support a role of Fe65 in DNA damage response in breast cancer cells, which have important implications for the therapeutic intervention of breast cancers with chemo- and radio-therapy that kills breast cancer cells by DNA damage.

7. Large amounts of Fe65 was detected in the cytoplasm of trip[le negative breast cancer cells that are usually more aggressive than ER-positive cells, suggesting that Fe65 may stimulate invasion/metastasis of triple negative breast cancers.

REPORTABLE OUTCOMES

1. Part of the data presented in this report was described in a poster presented in the 2011 Era of Hope Meeting:

   Yuefeng Sun, Jinfu Tang, Ming Hu, Ravi Kasiappan, Anfernee K-W Tse, Santo V. Nicosia, Zhang Mary Xiaohong Wenlong Bai. A NOVEL ROLE FOR Fe65 IN ESTROGEN RECEPTOR ACTION IN HUMAN BREAST CANCER CELLS, **2011 Era of Hope Meeting**, August 2 – 6, 2011, Orlando, FL.

2. Part of the data presented in this report was presented in a manuscript that has been drafted recently and will be submitted to the Journal of Biological Chemistry:

   Yuefeng Sun\, Jinfu Tang, Ming Hu, Ravi Kasiappan, Panida Lungchukiet, Waise Quarni, Xiaohong Zhang and Wenlong Bai. A Novel Function of the Fe65 Neuronal Adaptor in Estrogen Receptor Action in Breast Cancer Cells. **Journal of Biological Chemistry**. In submission.

   Both the abstract and the manuscript are enclosed with this report.

CONCLUSIONS

Our studies support our original hypothesis that Fe65 is a positive regulator of ERα action in breast cancer cells. Our studies support the following model of Fe65 action: Estrogens recruit Fe65 to its target gene promoters which in turn bind to ERα and increases its transcriptional activity by promoting its recruitment together with coactivators to the promoters of the same set of estrogen target genes such as Myc and IGF1, which both are known stimulators of breast cancer growth. Through its effect on ERα, Fe65 potentiates estrogen stimulation and reduces tamoxifen inhibition of breast cancer growth. Overall, the studies establish a neuronal adaptor with known functions in APP signaling in Alzheimer's disease as a positive regulator of breast cancer growth, providing a molecular link between neuro-degeneration disease and cancer. Althought not part of the original proposal, the studies may

"So what section": Our studies reveal a novel mechanism by which the molecules with neuronal functions regulate the actions of estrogens and antiestrogens in breast cancer cells. Our studies may identify Fe65-ERα complex as a potential molecular target for breast cancer therapy and prevention with anti-estrogens such as tamoxifen.

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APPENDICES: A meeting abstract and a manuscript are enclosed as appendices.

SUPPORTING DATA: All data were either described in yearly reports, embedded in the main body of this report or presented in the attached manuscript. No additional figures and/or tables are included as a separate section.

BIBLIOGRAPHY OF PUBLICATIONS AND ABSTRACTS:

1. *Yuefeng Sun, Jinfu Tang, Ming Hu, Ravi Kasiappan, Anfernee K-W Tse, Santo V. Nicosia, Zhang Mary Xiaohong Wenlong Bai.* A NOVEL ROLE FOR Fe65 IN ESTROGEN RECEPTOR ACTION IN HUMAN BREAST CANCER CELLS, **2011 Era of Hope Meeting**, August 2 – 6, 2011, Orlando, FL.

2. *Yuefeng Sun\, Jinfu Tang, Ming Hu, Ravi Kasiappan, Panida Lungchukiet, Waise Quarni, Xiaohong Zhang and Wenlong Bai.* A Novel Function of the Fe65 Neuronal Adaptor in Estrogen Receptor Action in Breast Cancer Cells. **Journal of Biological Chemistry**. In submission.

LIST OF PERSONNEL (NOT SALARIES) RECEIVING PAY FROM THE RESEARCH EFFORT:

1. *Wenlong Bai, Ph.D.*
2. *Ming Hu, Ph.D.*
3. *Jinfu Tang, Ph.D.*
4. *Yuefeng Sun, Ph.D.*

Meeting Abstract: 2011 Era of Hope Meeting, August 2 – 6, 2011, Orlando, FL

A Novel Role of Fe65 in Estrogen Receptor Action in Human Breast Cancer Cells

Authors: Yuefeng Sun, Jinfu Tang, Ming Hu, Ravi Kasiappan, Anfernee K-W Tse, Santo V. Nicosia, Xiaohong (Mary) Zhang and Wenlong Bai

Fe65 is a multiple-domain adaptor protein in the APP-Tip60 transcriptional complex well-known for its function in neuronal cells and neuro-degeneration diseases, but there are essentially no reports about its function in cancer cells. Our published data showed that Fe65 formed a complex with ERalpha in neuronal cells, which permits estrogen protection of neuronal cell apoptosis. Here we report our novel finding that Fe65 is expressed in breast epithelial and cancer cells and acts as an ERalpha coregulator. Our studies detected an increased expression of Fe65 in human breast cancer cells and breast tumors compared with controls. Fe65 transiently expressed in Fe65-negative Hela cells formed a protein complex with ectopic ERalpha and increased its transcriptional activity in reporter assays. Stable Fe65 expression in MCF-7 cells increased the activity of endogenous ERalpha as well as estrogen-induced target expression and cell growth. These results support our hypothesis that Fe65 plays an important role in estrogen action and breast tumorigenesis. To further define such a role for Fe65, we have constructed Tet-inducible Fe65 shRNA expression plasmids and transfected them into MCF-7 cells. Transgenic mouse lines with targeted Fe65 expression in mammary epithelial cell are also being produced. We expect that further studies will generate additional evidences fully supporting a positive role of Fe65 in estrogen stimulation of mammary tumorigenesis *in vivo*.

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A Novel Function of the Fe65 Neuronal Adaptor in Estrogen Receptor Action in Breast Cancer Cells*

Yuefeng Sun[a], JInfu Tang[a], Ming Hu[a], Ravi Kasiappan[a], Panida Lungchukiet[a], Waise Quarni[a], Xiaohong Zhang[a,b,c] and Wenlong Bai[a,b,c,1]

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Running title: *Fe65 and Breast Cancer*

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Key words: breast cancer, estrogens, estrogen receptor, Fe65, tamoxifen.

CAPSULE

Background: Fe65 is a neuronal adaptor for Aβ amyloid precursor protein and plays a role in the pathogenesis of the Alzheimer's disease.

Results: Fe65 is expressed in breast cancer cells and regulates promoter recruitments of estrogen receptors and coactivators and breast cancer growth response to estrogens and tamoxifen.

Conclusion: Fe65 is an important regulator of estrogen receptor action in breast cancer cells.

Significance: The studies define a novel role for a neuronal adaptor in estrogen actions in breast cancer cells.

SUMMARY

Fe65 is a multidomain adaptor with established functions in neuronal cells and neuro-degeneration diseases. It binds to the carboxyl terminus of the Aβ amyloid precursor protein (APP) and is involved in transcriptional regulation. Our published studies have shown that Fe65 forms a complex with the estrogen receptor alpha (ERα), which permits estrogen protection of neuronal cells from apoptosis induced by the APP transcriptional complex. Here we report our new finding that Fe65 is expressed in breast cancer (BCa) cells and acts as an ERα transcriptional coregulator that is recruited by 17β-estradiuol to the promoters of estrogen target genes. Deletion analyses mapped the ERα binding domain to the phosphotyrosine binding domain 2 (PTB2). Ectopic Fe65 increased the transcriptional activity of the ERα in a PTB2 dependent manner in reporter assays. Fe65 knockdown decreased and its stable expression increased the activity of endogenous ERα in breast cancer cells and estrogen-induced target gene expression, recruitments of the ERα and coactivators to target gene promoters and cell growth. Furthermore, Fe65 expression decreased the antagonistic activity of tamoxifen, suggesting a role for Fe65 in tamoxifen resistance. The studies define a novel role for a neuronal adaptor in estrogen actions in breast cancer cells.

INTRODUCTION

Estrogens are the female sex steroid hormone with established roles in reproduction, development as well as the biology and pathogenesis of many tissues including those of the central nervous, the cardiovascular, and the skeletal systems, etc. (1,2). The best defined estrogen target tissues include the mammary gland of which both the normal development and epithelial tumorigenesis are subjected to estrogen regulations. Due to the retained sensitivity of the majority of breast cancers (BCa) to estrogens, the inhibition of estrogen action at the tumor with synthetic antagonists such as tamoxifen has been the preferred therapeutic treatment for decades (3,4). The effects of estrogens and antiestrogens on mammary epithelial and cancer cells are predominantly mediated through the estrogen receptor alpha (ERα) that belongs to the steroid/thyroid nuclear receptor superfamily of ligand-regulated transcription factors (5,6). The

ERα contains an N-terminal A/B region, a DNA-binding domain (DBD) composed of two C_2C_2 zinc fingers, a hinge region, a ligand-binding domain (LBD) and an F tail (5). It forms a homodimer and binds to estrogen response elements (ERE) to control target gene expression. In addition, the ERα also regulates target gene expression through its "tethering" to other transcription factors (7,8). In response to estrogens, the ERα recruit coactivators (9) to induce growth promoting genes are together with other co-regulators. The binding of estrogen antagonists on the other hand induces a distinct conformation that triggers the binding of corepressor complexes including NCoR and SMRT, thereby shutting off gene transcription (10,11). Tamoxifen (TAM), which aims to block ERα action, has mixed agonist/antagonist activity and may either stimulate or antagonize ER function in tissues and genes (12). The use of TAM has benefitted women with ERα positive breast cancers but been met with resistance.

Besides mammary gland development and tumorigenesis, another known target for estrogens is the brain. Estrogens have been shown to be neuroprotective and their decrease after menopause is believed to contribute to the development of neurodegenerative diseases such as Alzheimer's Disease (AD). APP plays important roles in the pathogenesis of AD and recent studies have shown that its C-terminal fragment produced after the cleavage by γ-secretase, namely APPct or AICD, forms a multimeric complex with the nuclear adaptor protein FE65 and stimulates transcription through the recruitment of the histone acetyl transferase Tip60 and the transcription factor CP2/LSF/LBP1 (13-15). Published studies have shown that 17β-estradiol inhibits the transcriptional activity of the APPct complex and impaired the ability of the complex to induce apoptosis of neuroblastoma cells (16), providing an mechanism explain the neuronal protective effects of estrogens. Both *in vitro* and *in vivo* immunological analyses have revealed that the ERα formed a complex with full length APP or APPct and that the interactions occur between endogenous proteins in mouse brains and were increased in the brains of transgenic mice expressing both mutant presenilin 1 and APP (16). Detailed mechanistic investigations have found that the functional interaction between ERα and APP is indirectly mediated through the APP adaptor FE65, identifying it as a novel ERα interacting protein (16).

Fe65 is a multidomain adaptor protein containing an undefined N-terminus, a group II tryptophan-tryptophan (WW) domain in the middle and two C-terminal PTB domains, namely PTB1 and PTB2 (17). Through PTB2, it forms a multimeric complex with APP or APPct to stimulate transcription through the recruitment of the transcription factor CP2/LSF/LBP1 and the histone acetyl transferase Tip60 (13-15) to PTB1 as well as the nucleosome assembly factor SET to the WW domain (18). The PTB1 domain also interacts with two cell surface lipoproteins receptors, the low-density lipoprotein receptor related protein (LRP) (19) and ApoEr2 (20), forming trimeric complexes with APP, which establishes a biological linkage between APP and the lipoprotein receptors. Besides SET, the WW domain also binds to Mena (21), through which it functions in regulating the actin cytoskeleton, cell motility, and neuronal growth cone formation (22,23).

There are two Fe65 isoforms produced by the alternative splicing of a 6-bp mini-exon encoding Arg-Glu dipeptide inserted in the PTB1 domain. The isoform with this mini-exon is expressed exclusively in neurons whereas the isoform lacking the dipeptide is detected in nonneuronal cells (24). Besides its neuronal functions in APP processing and AD biology, Fe65 has been reported to regulate other essential cellular functions such as DNA damage repair that goes beyond neuronal cells. Fe65 null mice are more sensitive to DNA damages induced by etoposide and ionizing radiations (25). Studies with Fe65 null mouse embryonic fibroblasts concluded that Fe65 was required for the efficient repair of DNA double strand breaks, a function that depends on its interaction with Tip60 and APPct (26,27). However, functions of Fe65 in non-neuronal cells are largely undefined and nothing is known about its involvement in estrogen actions in breast cancers.

In the present study, we demonstrate for the first time that Fe65 is expressed in mammary epithelial cells and that its expression is increased in breast cancer cells and human breast tumor samples. Fe65 is recruited by estrogens to the promoters of ERα target genes in breast cancer cells and potentiates the recruitment of ERα and its coactivators to the promoters. It increases the agonistic activity of 17β-estradiol and decreases the antagonistic activity of tamoxifen. The data define Fe65 as a positive ERα regulator that stimulates the growth of human breast cancer cells and may contribute to tamoxifen resistance.

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EXPERIMENTAL PROCEDURES

Reagents and antibodies - 17β-estradiol (E2758), Anti-Flag Affinity gels (A2220) and Thiazolyl Blue Tetrazolium Bromide (MTT, M2128) were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) (Cat, 10082-147), charcoal stripped FBS (cFBS) (Cat, 12676-029) and lipofectamine 2000 were bought from Invitrogen (Grand Island, NY). Anti-hemagglutinin (anti-HA.11; PRB-101P) antibody was obtained from Covance (Princeton, NJ). Anti-Fe65 (#2877), Anti-c-Myc (#9402), Anti-Cyclin D1 (#2922) were purchased from Cell Signaling Company (Boston, MA). The following antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA): Anti-ERα (F10, sc-8002), Anti-β-Actin (AC-15, sc-69879), Anti-HSP60 (H-1, sc13115), HDAC1 (H-11, sc8410), Anti-Tip60 (N-17, sc-5725), Anti-Histone H1 (N-16, sc-34464x). Fe65 (siFe65) (sequence: CUACGUAGCUCGUGAUAAG) and scrambled control (siCtrl) siRNA were obtained from Dharmacon/Thermo Scientific (Waltham, MA). The ECL Western blotting substrates were obtained from Thermo Scientific. Luciferase assay substrates were obtained from Promega Corporation (Madison, WI). Chip assay kit (EZ-Chip™, 17-371) was purchased from Millipore (Billerica, MA). Pairs of breast tumor and adjacent normal tissue samples were obtained from the tissue procurement facility at H Lee Moffitt Cancer Center and their usage was approved by the institutional review board at University of South Florida.

To construct tagged Fe65, cDNA of the non-neuronal Fe65 (Thermo Scientific, Waltham, MA) was amplified by polymerase chain reactions using forward (GCGGGATCCATGTCTGTTCCATCATCAC TG) and reverse (GAGGTCGACTCATGGGGTATGGGCCCC) primers. Myc-Fe65 was constructed by cloning the amplified cDNA into the Bam H1 and Sal1 of p-CMV-3Tag-2a-Myc plasmid (Agilent Technologies, Santa Clara CA). To construct the expression plasmids of HA-Fe65 and deletion constructs, Fe65 cDNA or fragments were amplified by polymerase chain reactions using the Myc-Fe65 as the DNA template, digested with BamH1 and Sal1 and then ligated into pCMV-HA plasmid generated by replacing the Flag tag with HA of pCMV-3Tag-1A (Agilent Technologies, Santa Clara CA). The primers used for the constructions of Fe65 deletion mutants are as follows: full length: GCGGGATCCATGTCTGTTCCATCATCACT GAGC (forward) and GAGGTCGACTCATGGGGTATGGGCCCCA GCCG (reverse); N-terminal 128 amino acid deletion (dN128): GCGGGATCCATGAACCGAGGCCTACGAG GACCT (forward) and GAGGTCGACTCATGGGGTATGGGCCCCC AGCCG (reverse); N-terminal 242 amino acid deletion (dN242): GCGGGATCCATGTTCTGGAACCCCAACG CCTTC (forward) and GAGGTCGACTCATGGGGTATGGGCCCCC AGCCG (reverse); WW deletion (dWW): TTCACCGGTCAAGAGGAGTCCCAGCTCA C (forward) and CTTACCGGTGTCGGAATCCGTCTCGAAG (reverse); PTB1 deletion (dPTB1): CCCAAGAGGAGGAGAAGTGCTTGGTAAA TGGACT (forward) AGTCCATTTACCAAGCACTTCTCCTCCTC TTGGG (reverse); PTB2 deletion (dPTB2): CTTGTGGATGTCCCTTTCCAATCCCAGGC CTC (forward) and GAGGCCTGGGATTGGAAAGGGACATCCA CAAG (reverse); C-terminal 182 amino acid deletion (dC182): GCGGGATCCATGTCTGTTCCATCATCACT GAGC (forward) GAGGTCGACTCATTGGAAAGGGACATCC

ACAAG (reverse); C-terminal 42 amino acid deletion (dC182): GCGGGATCCATGTCTGTTCCATCATCACT GAGC (forward) and GAGGTCGACACGGGCATCCAGACACTTC TGGTA (reverse).

Flag-ERα plasmid was a gift from Dr. Muyan (28). pGEX-ERα(297-595) was provided by Dr. Corbo (29) and AIB1 by Dr. Melzer (30). pLENβgal and EREe1bLuc plasmids had been used in our previous studies (16,31).

Cell culture - MCF-7, T47D, MDA-MB-231 and MDA-MB-361 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% FBS. Mouse mammary tumors cells derived from $VDR^{+/+}$ (WT145) and $VDR^{-/-}$ (KO240) were kindly provided by Dr. Welsh (32) and maintained in DMEM/F-12 medium as described. BT474 and ZR75-1 cells were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% FBS. MCF-10A cells were maintained in DMEM/F-12 medium containing 10 µg/mL Insulin, 20 ng/mL EGF, 100 ng/mL Cholera toxin, 0.5 µg/mL hydrocortisone, 100 units/ml penicillin, 100 µg/ml streptomycin, and 5% FBS.

Transfections, Fe65 knockdown and reporter assays – For reporter assays, cells were starved for 3 days in DMEM containing 3% cFBS and transfections were performed with lipofectamine 2000. Transfected cells were treated with vehicle (ethanol, EtOH) or 17β-estradiol for 48 hours (h). Cellular extracts were prepared by directly adding lysis buffer containing 25 mM Tris-phosphate (pH 7.8), 2 mM dithiothreitol, 2 mM 1, 2-diaminocyclohexane-N,N,N',N'-tetyraacedtic acid, 10% glycerol, and 0.2% Triton X-100. Luciferase and β-galactosidase activity was determined as previously described (31,33).

For Fe65 knockdown, cells were transfected with Fe65 or control siRNA and 24 h post-transfection, Fe65 knockdown was verified by immunoblotting (IB). For Fe65 stable expression, MCF-7 cells were transfected with Myc-Fe65 or empty vectors and stable clones selected in the presence of 1 µg/ml puromycin. Individual clones were isolated and Fe65 expression was confirmed by IB. For ERα target gene expression, transfected cells were treated with 17β-estradiol for 2 h and the expression of selective estrogen target genes were measured by IB and quantified by ImageJ (NIH, http://rsb.info.nih.gov/ij/).

GST pull-down assays - pGEX-ERα(297-595) in which GST fused with C-terminal ligand binding domain of the ERα (29) was transformed into the BL21(DE3) *Escherichia coli* strain. Transformed bacteria were cultured at 37°C until the optical density at 600 nm reached 0.8, and the culturing was continued for 4 hours in the presence of 0.5 mM isopropylthiogalactopyranoside (IPTG). The bacteria were harvested and sonicated in buffer containing 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 6 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. GST fusion proteins were purified by binding to glutathione-Sepharose 4B beads (Amersham Pharmacia/GE Health Care, Piscataway, NJ). Then, 50 µg of bead-bound GST-ERα were incubated overnight at 4°C with extracts of 293T cells transfected with Fe65 constructs and washed with the lysis buffer containing 0.5% NP-40. Fe65 proteins bound to the beads were released by boiling in SDS-polyacrylamide gel (SDS-PAGE) sample buffer, resolved in an 8% SDS-polyacrylamide gel and detected by IB analyses.

Subcellular fractionation - Subcellular fractionation was carried out as described previously (34) with minor modifications. Briefly, the cell pellets were re-suspended in buffer containing 10 mM HEPES-NaOH (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol and incubated on ice for 15 min. To 400 µl of the suspension solution, 12.5 µl of 10% (v/v) Nonidet P-40 was added, followed by agitation for 10 seconds and centrifugation at 13, 000 rpm for 1 min. The supernatant was collected for cytoplasm proteins and the pellet (crude nucleus fraction) was washed 3 times with the same buffer containing 0.625% (v/v) Nonidet P-40 and re-suspended in the extraction buffer containing 10 mM HEPES-NaOH (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, put on ice for 30 min and vortex every 5 min, centrifuge for 5 min at 13,000 rpm, collect the supernatant as nuclear proteins. All

the samples (cytoplasm fraction and nuclear fraction) were analyzed by IB.

Immunological analyses - For immunoprecipitations (IP), cells were suspended in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% (v/v) NP-40, 1 mM PMSF, protease inhibitor cocktail and sonicated for 6 seconds with two repeats, then put on ice for another 10 minutes. After centrifugation, cellular extracts were incubated with primary antibodies overnight at 4°C. The beads were washed with cold lysis buffer for three times and boiled in SDS–PAGE sample buffer. Eluted samples were then subjected to IB analyses.

For IB analyses, precipitates or cellular extracts were separated in SDS-PAGE, transferred to a nitrocellulose membrane, and probed with cognate antibodies. Horseradish peroxidase-linked secondary antibodies and enhanced chemiluminescence reagents (Thermo Scientific, Waltham, MA) were used to detect the proteins.

For chromatin IP (ChIP) assays, cells were plated in DMEM medium containing 10% FBS. One day after plating, the medium was changed to DMEM containing 3% cFBS for estrogen deprivation. For T47D cells, siRNA transfections were performed with lipofectamine 2000 24 h after estrogen depletion. Then, cells were cultured in DMEM medium containing 3% charcoal-stripped FBS for another 48 h before estrogen treatment. For MCF-7 stable clones, cells were starved for 72 h. After estrogen deprivation, cells were treated with ethanol or 1×10^{-7} M 17β-estradiol for 45 min before cross-linking with 1% formaldehyde. All subsequent steps followed the company's instructions (EMD Millipore, Billerica, MA). ChIP assay primers for cathepsin D, c-Myc and IGF-1 had been described previously (35): The primers for EBAG9 were as follows: ATTGTCTGCCCTTCGCCGT (forward) and TTTGGAGGCTGCGTGCTTT (reverse).

MTT assays - Cells were seeded into 96-well plates at a density of 2×10^{3} cells per well. 24 hours later, cells were treated with ethanol, 1×10^{-8} M 17β-estradiol or 10^{-7} M tamoxifen (TAM) for various times. MTT reagent was added to each well to give a final concentration of 0.5 mg/ml and incubated for 3 h. After removing the medium, 100 µl DMSO was added and the absorption at 595 nm was determined with a MRX microplate reader (DYNEX Technologies, Chantilly, VA).

RESULTS

Fe65 is a positive ERα transcriptional regulator – Published studies have shown that estrogens regulate the transcriptional activity of APPct through protein complex formation between Fe65 and the ERα detected both *in vitro* and in mouse brain (16). The complex formation raises the possibility for Fe65-APPct complex to regulate ERα activity. To test this idea, Fe65 and ERα were ectopically expressed in ERα and Fe65-negative Hela cells and ERα transcriptional activity was measured with co-transfected EREe1bluc reporter genes. As shown in Fig 1A, ectopic Fe65 expression in Hela cells increased the ERα transcriptional activity induced by 17β-estradiol in a dose-dependent manner. The increase was about half of what was induced by AIB1 and nevertheless statistically significant. In similar assays, ectopic APPct expression did not change ERα activity, either in the presence (Fig 1B) or absence (Fig 1C) of Fe65. The analyses identify Fe65 as a positive ERα regulator that stimulates ERα activity in a manner that appears to be independent of its complex formation with APPct.

Fe65 binds to ERα and stimulates its activity through the PTB2 domain - Previous studies defined the Fe65 binding domain to the C-terminal ligand binding domain of the ERα (16). To define the Fe65 domain mediating its interaction with the ERα, Fe65 mutants lacking defined domains were generated (Fig. 2A) and their ability to bind to ERα assessed in co-IP analyses (Fig. 2B) and GST pull-down assays (Fig. 2C). In cells co-transfected with Flag-ERα with either Myc or HA-tagged full length Fe65, Fe65 was detected in Flag precipitates together with ERα. No Fe65 was detected in the precipitates when cells were transfected with control vector and Myc-Fe65, showing that the presence of Fe65 in the Flag precipitates accurately measured the interaction between ERα and Fe65. Deletion of Fe65 regions containing PTB2 eliminated its ability to co-precipitate Flag-ERα (Fig. 2A) whereas the

deletion of other regions had little effects, indicating that the interaction mediated through PTB2. This conclusion was confirmed by subsequent GST pull down assays (Fig 2C), in which GST-ERα-C-terminus (GST-ER-C) containing the LBD precipitates all tested Fe65 fragments except the one lacked PTB2. Overall, the co-precipitation analyses suggest that the interaction is mediated through direct interaction between the Fe65 PTB2 domain and the ERα LBD.

Consistent with the mapping analyses, reporter assays showed that the PTB2 domain is essential for Fe65 to potentiate ERα transcriptional activity. As shown in Fig. 3, while full length Fe65 increased ERα activity in a dose-dependent manner, the PTB2 deletion mutant did not change ERα activity in in parallel analyses (Fig 3A). IB analyses showed that the PTB2 deletion mutant was expressed to the levels comparable to the full length protein, ruling out the possibility that the lack of an effect of the PTB2 mutant is due to the inability of the vector to efficiently express the protein. Levels of the ERα protein expression were not altered by the full length Fe65 or the PTB2 deletion mutant, showing that the reporter analyses truthfully reflected their effect on the specific activity of ERα per molecule. The reporter analyses, together with the domain mapping, allow the conclusion that Fe65 binds to the ERα and potentiate its activity through the PTB2 domain.

Fe65 is expressed in mammary epithelial cells and the expression increased in breast cancer cells and tumor tissues - ERα plays an important role in breast cancer development and mediates the mitogenic activity of estrogens in stimulating breast cancer growth. The interaction between non-neuronal from of Fe65 with the ERα suggest a possible role for Fe65 in estrogen action in breast cancers. Consistent with this idea, Fe65 was detected in the non-tumorigenic MCF-10A human breast epithelial cells and its expression was found to be increased in human breast cancer cells as well as two mouse mammary tumor cells (WT145 and VDR-KO) (Fig. 4A). The increased expression was detected in both ERα-negative and positive cells with the exception of MCF-7. Cellular fractionation revealed the presence of Fe65 in the nucleus of all tested breast cancer cells, including MCF-7 (Fig. 4B), which was apparently absent in MCF-10A cells. Consistent with the data in cell lines, Fe65 expression was found to be expressed at higher levels in about 60% of human breast tumor samples as compared to cognate normal controls (5 out of 8) (Fig, 4C). The increased Fe65 expression in breast cancers, particularly the increase in the nucleus, makes it possible for Fe65 to act as a positive regulator of estrogen action in breast cancers to promote breast tumorigenesis.

Fe65 is a positive regulator of endogenous ERα in breast cancer cells, which is recruited by estrogens to ERα target gene promoters and in turn, facilitates the recruitments of ERα and its coactivators – To test its effect on endogenous ERα, Fe65 was either stably over expressed or knocked down in ERα-positive breast cancer cells and the changes in the transcriptional activity of endogenous ERα was determined with transfected EREe1bluc reporters. The transcriptional analyses showed that stable Fe65 expression in MCF-7 cells significantly increased estrogen stimulation of endogenous ERα activity was in a dose-dependent manner (Fig 5A) whereas Fe65 knockdown in both T47D (Fig. 5B) and BT474 (Fig. 5C) cells decreased ERα activity. It is important to note that, under our assay conditions, the activity of endogenous ERα on transfected EREe1bluc reporter is weaker than ectopic ERα expressed in Hela cells, which may explain the weaker effects of Fe65 on endogenous ERα in breast cancer cells observed in reporter analyses.

Consistent with the reporter based transcriptional analyses, transient transfection of Fe65, but not the PTB2 deletion mutant, into MCF-7 cells increased estrogen induction of c-Myc and cyclin D1 in a dose-dependent manner (Fig 6A), suggesting that the positive effect of Fe65 on endogenous ERα observed in reporter analyses can be translated into changes in ERα target gene expression and that the effect on the target genes likely involves PTB2-mediated complex formation with endogenous ERα. Similarly, stable Fe65 expression increased (Fig. 6B) whereas Fe65 knockdown in T47D (Fig. 6C) and BT474 (Fig. 6D) decreased the ability of estrogens to induce ERα target gene

expression. The levels of endogenous ERα protein expression were not altered by either Fe65 over expression or knockdown, suggesting that the positive effect is likely due to an increase in ERα activity. The data demonstrate that the Fe65 is a positive regulator of the endogenous ERα in breast cancer cells that exerts the positive effect through the PTB2-mediated complex formation with ERα without an effect on its expression.

To understand how Fe65 exerts its positive effects on endogenous ERα in breast cancer cells, ChIP assays were performed to test whether Fe65 is recruited to the promoters of ERα target genes and more importantly, whether changes in levels of Fe65 expression alter the promoter recruitment of ERα and its coactivators. As shown in Fig 7A, 17β-estradiol recruited Fe65 to the promoters of 4 different ERα target genes in T47D cells and the amounts of Fe65 on the promoters was decreased by Fe65 siRNA. Interestingly, Fe65 knockdown also resulted in decreased recruitments of both ERα and its coactivator AIB1 to the promoters of all 4 tested target genes, showing a fundamental role of Fe65 in determining the chromatin recruitment of the ERα and its coactivators. Such a role for Fe65 was further supported by the finding that the stable Fe65 expression in MCF-7 cells increased the estrogen recruitment of ERα and AIB1 to target gene promoters (Fig 7B). More importantly, the ability of tamoxifen (TAM) to block estrogen-induced ERα and AIB1 recruitment to promoters was significantly suppressed by Fe65 stable expression in MCF-7 cells, suggesting a potential role for Fe65 in TAM resistance.

The increased ERα and AIB1 recruitment to target gene promoters can be easily explained if Fe65 promotes estrogen-induced ERα nuclear localization. To test this, cellular distribution of ERα and Fe65 was analyzed with nuclear and cytosolic extracts prepared from control and Fe65 stable clones of MCF-7 cells. As shown in Fig 7C, the stable expression of Fe65 resulted in a significant increase of its presence in both the cytosol and the nucleus. However, the Fe65 stable expression did not alter ERα localization to nucleus induced by 17β-estradiol, showing a lack of Fe65 effect on ERα nuclear localization. Overall, the data suggest that the positive effect of Fe65 on ERα recruitment to target genes promoters is likely due to a fundamental role of Fe65 in facilitating ERα to bind chromatin of target gene promoters.

Fe65 promotes estrogen induced breast cancer cell growth and suppresses the antagonistic activity of TAM - Given that ERα activity and the expression of c-Myc and cyclin D1 are often associated with cell growth in breast cancer cells, the positive effects of Fe65 on ERα activity and target gene expression should translate into a positive effect on breast cancer cell growth and its stimulation by estrogens. Indeed, our cell growth analyses showed that Fe65 knockdown significantly decreased the ability of 17β-estradiol to promote cell growth of T47D (Fig. 8A) and ZR75-1 (Fig. 8B) cells. Consistently, the ability of 17β-estradiol to stimulate MCF-7 cell growth was significantly higher in Fe65 stable clones in comparison to control clones (Fig 8C). The increasing in estrogen stimulation is positively correlated with Fe65 expression levels in the stable clones.

Consistent with the ChIP assay data presented in Fig. 7B, the ability of TAM to suppress cell growth stimulation by 17β-estradiol was significantly decreased by the stable Fe65 expression (Fig. 8D). In the control clone, TAM blocked 17β-estradiol-induced cell growth by about 81.6% whereas in the Fe65 overexpression clone, the inhibition was reduced to 61.7%. The data clearly demonstrated that Fe65 can promote estrogen-induced cell growth and reduces the antiestrogenic effect of TAM in suppressing estrogen induced growth of breast cancer cells.

DISCUSSION

Fe65 has been well studied in Alzheimer's disease (AD) for its functions in mediating the trafficking and processing of APP as well as its participation in transcriptional regulation (36). Up to now, there are essentially no reports about the function of Fe65 in cancer cells. The present studies are the first to clearly establish a role for Fe65 in breast cancers. Multiple lines of evidences are presented to support the novel role of Fe65 as a positive ERα regulator that potentiate estrogen stimulation of

breast cancer cell growth. First, Fe65 bound to the ERα through the PTB2 domain and increased ERα activity in reporter assays in a PTB2 dependent manner. Secondly, Fe65 expression was detected in breast epithelial cells and its expression was found to be increased in breast cancer cells and tumor tissue samples. Thirdly, 17β-estradiol recruited Fe65 to the promoters of natural estrogen target genes. Finally, Fe65 stable expression increased and its knockdown decreased the activity of endogenous ERα in breast cancer cells as well as estrogen-induced target gene expression, recruitment of ERα and coactivators to target gene promoters and cell growth. Together with studies published previously (16), the present studies support the extension of Fe65 action from neurons to breast cancer cells as presented in Fig. 8E. In neuronal cells, Fe65 brings the ERα to APPct transcriptional complex, allowing estrogens protection of neuronal cells from apoptosis induced by toxicity as a result of APP cleavage. In breast cancer cells, Fe65 binds to the ERα and potentiates its chromatin binding at target gene promoters, which permits Fe65 to act as a positive stimulator of breast cancer cell growth. Although it remains to be determined, the increased Fe65 expression in breast tumor tissue samples suggest that the findings about Fe65 in breast cancer cell lines will most likely reflect an *in vivo* role for this neuronal adaptor in breast tumorigenesis.

Tip60 is a histone acetyl transferase that binds to PTB1 domain of Fe65 (13). It was also reported to be a coactivator for the ERα (37). It is a legitimate question whether the positive effect of Fe65 on the ERα involves Tip60. Our studies have yielded conflicting data regarding the role of Tip60 in ERα activation by Fe65. In ChIP assays, estrogens recruited Tip60 to ERα target gene promoters, which were enhanced by Fe65 over expression (data not shown). The data are consistent with the fact that Tip60 is a common interacting protein for both the Fe65 and the ERα. However, in reporter gene analyses, the ectopic expression of Tip60, instead of synergizing with Fe65 in stimulating ERα activity as it would be expected for a coactivator, decreased the ability of Fe65 to activate the ERα (data not shown). The reporter data are consistent with the fact that APPct did not increase ERα activity or potentiate the positive effect of Fe65 on the ERα (Fig. 1). Tip60 has been shown to be a tumor suppressor of which the expression, particularly the expression in the nucleus, is decreased on breast tumors (38). Interestingly, it has been shown that estrogen-induced c-Myc and cyclin D1 mRNA expression were not affected by Tip60 depletion in MCF-7 cells (39) even though several non-growth related ERα target genes were altered, suggesting a selective involvement of Tip60 in ERα action in breast cancer cells.

Our data suggest that APPct is not involved in ERα activation by Fe65 (Fig. 1). Questions remain what signals regulate Fe65 nuclear localization in breast cancer cells and its positive effect on ERα. Under over expression conditions, Fe65-ERα complex formation appeared to be constitutive and not changed by estrogen treatments (data not shown). However, ChIP assays revealed that the Fe65 recruitment to ERα target gene promoters were induced by 17β-estradiol (Fig. 7), suggesting that estrogens are part of the signals operating in breast cancer cells to regulate the actions of Fe65-ERα complex in the nucleus. Fe65 over expression increased the Fe65 levels both in the cytoplasm and in the nucleus of in MCF-7 cells (Fig. 7C), showing that a simple way of promoting nuclear Fe65-ERα activity is to increase Fe65 expression, which naturally occurs in breast tumors as suggested by data in Fig. 4C. In addition to APP cleavage, Fe65 phosphorylations had been shown to induce Fe65 nuclear localization (40). Fe65 has also been reported to interact with the C-terminus of Notch1 (41). It is important to find out whether these signaling pathways known to be active in breast cancers regulate ERα activation through Fe65.

Although the present studies focused on the effect of Fe65 on ERα actions, it is important to point out that Fe65 may have a much broad impact on breast cancer biology beyond estrogen stimulation of cell growth. For example, Fe65 has been described to be required for efficient repair of DNA double strand breaks, a function that depends on its interaction with Tip60 and

APPct (26,27). There are also reports showing that the phosphorylated H2A.X is higher in Fe65 null cells than the wild type under genotoxic damages (25) and that the phosphorylation is somehow dependent on Fe65 accumulation in the nucleus (42-44). Thus, through Fe65-ERα complex formation, estrogens may regulate DNA damage repair in breast epithelial cells. In addition, the WW domain of Fe65 is known to bind to Mena (21), through which it regulate the actin cytoskeleton, cell motility, and neuronal growth cone formation (22,23). Fe65 thus may also control breast cancer invasion through similar mechanism and allow estrogens to promote breast cancer invasion through the Fe65-ERα complex. In this regard, it is important to point out that the majority of the Fe65 signals was detected in the cytoplasm of breast cancer cells and that the cells expressing the highest amounts of Fe65 were triple-negative breast cancer cells which are more aggressive.

In summary, the present studies have extended the functions of the Fe65 neuronal adaptor protein to breast cancer cells and defined a role for Fe65 in estrogen action and breast cancer cell growth. The existence of Fe65-ERα complex in breast cancer cells increased the complexity of the mechanisms underlying estrogen actions in breast cancer cells. For the reason that Fe65 is a multi-domain protein that interacts with multiple proteins with diverse functions, its interaction with ERα may change the receptor activity both quantitatively and qualitatively by allowing more complex regulation patterns. Our finding that Fe65 over expression suppressed the antiestrogenic activity of TAM implicates that Fe65 may be a new molecular target for breast cancer intervention and that targeted disruption of the ERα-Fe65 interaction may represent a new strategy to overcome TAM resistance.

REFERENCES

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Fe65 potentiates estrogen activation of the ERα. (A), Hela cells were plated in 12-well plates and transfected with 0.1 μg EREe1bluc, 0.1 μg pLENβgal and 0.2 μg Flag-ERα, together with indicated amounts of HA-Fe65 or 0.3 μg AIB1 and treated with vehicle (EtOH) or 10^{-8} M 17β-estradiol (E2) for 48 h. Luciferase activity was determined and normalized with β-gal activity. Folds of ERα activity was calculated by dividing the normalized luciferase activity with the activity of control cells transfected with empty vector and treated with EtOH. **(B),** Hela cells were transfected as in panel A but with indicated amounts of APPct. Cells were treated and ERα activity was determined and normalized similarly. **(C)**, Hela cells were plated and transfected as in panel A but with indicated amounts of Fe65 and APPct. Cells were treated and ERα activity was determined and normalized similarly. Each reporter data point

represents analyses in triplicate performed in parallel and each experiment was repeated three times. The error bars stand for standard deviation (SD). Statistical analyses were performed with student's *t* test (n=9).

Fig. 2. Fe65 PTB2 domain mediates its interaction with the ERα. (A), Schematic representations of the domain structure of Fe65 and deletion mutants. The numbers of amino acid residues of full length Fe65 and its domains are marked. **(B),** 293T cells were transfected with 1.5 μg Flag-ERα and 1.5 μg of Myc (left panels) or HA-tagged (right panels) Fe65 or deletion mutants as indicated. Cellular extracts were made and subjected to IP/IB analyses with antibodies as indicated. **(C),** GST and GST-ERα-C was produced in and purified from bacteria and incubated lysates of cells transfected with HA-tagged Fe65 deletion mutants. GST pull-down assays were performed and proteins in the precipitates were detected by IB with anti-HA antibody (lower panels). Coomassie blue (C.B.) staining was included to show that comparable amounts of GST and GST-ER-C were used in the pull-down assays. IB analyses of the lysates were also included to show the expression levels of the Fe65 deletion mutants.

Fig. 3. The PTB2 domain is essential for Fe65 to potentiate estrogen stimulation of ERα transcriptional activity. (A) Hela cells were plated and transfected as in Fig 1A but together with indicated amounts of HA-tagged Fe65 or PTB2 deletion mutant. Cells were treated and ERα activity was determined and normalized similarly. **(B)**. IB analyses were performed in parallel with lysates from transfected cells to show that Fe65 and the PTB2 deletion mutant were expressed at comparable levels and did not change ERα protein expression. β-actin blot was included to show even loading.

Fig. 4. Fe65 is expressed in breast epithelial cells and the expression is increased in breast cancer cells and tumor tissue samples. (A) Whole cell lysates of non-tumorigenic breast epithelial (MCF-10A) and cancer cells were subjected to IB analyses with antibodies as indicated. **(B)** Cytoplasmic and nuclear extracts of breast cell lines were subjected to IB analyses with antibodies as indicated. Hsp60 and HDAC1 immunoblots were included to show the separation of cytoplasmic and nuclear extracts. β-actin blot was included to show even loading. **(C)** Extracts of paired human breast tumor (T) and adjacent normal (N) tissues were assayed for total proteins, of which equal amounts were subjected to IB analyses with anti-Fe65 antibody. The signal of Fe65 and a nonspecific protein on the blot was quantified using the ImageJ software (Version 1.46) from NIH. The ratio of normalized Fe65 signal (tumor divided by normal) was plotted.

Fig 5. Fe65 potentiates estrogen stimulation of endogenous ERα activity in breast cancer cells. (A) MCF-7 cells stably transfected with empty vector (Ctrl) or Fe65 (Fe65 OE clones) were further transfected with 0.3 μg EREe1bluc and pLENβGal. **(B)** and **(C),** T47D (panel B) and BT474 (panel D) cells were transfected with siCtrl or siFe65 and 24 h later, re-transfected with 0.3 μg EREe1bluc and PlemβGal. Transfected cells were treated and luciferase activity was determined and normalized as in Fig 1A. Fe65 over expression and knockdown as well as its effect on ERα protein expression was monitored by IB analyses with indicated antibodies**.**

Fig 6. Fe65 potentiates the estrogen induction of ERα target gene expression in breast cancer cells. (A) MCF-7 cells were estrogen starved for 24 h and transfected with empty vector (Ctrl), different amounts of Fe65 or PTB2 deletion mutant and 48 h later, subjected to estrogen treatment. **(B)** MCF-7 cells stably transfected with empty vector or Fe65 were estrogen starved for 72 h and subjected to estrogen treatment. **(C)** and **(D)**, T47D (panel C) and BT474 (panel D) cells were transfected with siCrtl or siFe65 and estrogen starved for 72 h. Transfected cells were treated with EtOH or 10^{-8} M E2 for 2 h. Cellular extracts were subjected to IB analyses with indicated antibodies. The expression levels of cyclin D1 and c-Myc were quantified with ImageJ and normalized with cognate β-actin signals. The normalized

signals were divided by the signal of control cells (transfected with empty vector or scrambled siRNA and treated with EtOH), which were shown at the bottom of the blots.

Fig 7. Fe65 is recruited to ERα target gene promoters by estrogens and required for optimal recruitment of the ERα and its coactivators. (A) T47D cells were estrogen starved for 24 h and transfected with siCtrl or siFe65. 48 h post transfection, cells were treated with EtOH or 10^{-7} M E2 for 45 min and ChIP assays were performed with indicated antibodies. **(B)** Control MCF-7 and Fe65 overexpression (Fe65 OE) clone (2-5) were estrogen starved for 72 h and treated with EtOH, 10^{-7} M E2 or 10^{-7} M E2 plus 10^{-7} M tamoxifen (TAM) (E2+TAM) for 45 min. ChIP assays were performed with indicated antibodies. **(C)** Control MCF-7 and Fe65 OE clone (2-5) were estrogen starved for 72 h, and treated with EtOH or 10^{-8} M E2 for 1 h. Cytoplasm and nucleus proteins were separated and subjected to IB analyses with antibodies as indicated. Hsp60 and HDAC1 immunoblots were included to show the separation of cytoplasmic and nuclear extracts.

Fig 8. Fe65 promotes estrogen-induced breast cancer cell growth and suppresses the antagonistic activity of tamoxifen. T47D **(A)** and ZR75-1 **(B)** cells were estrogen starved and transfected with siCtrl or siFe65 and 48 h later, re-transfected for another time. 24 h post the 2nd transfection, the cells were plated in 96-well plates and treated with EtOH or 10^{-8} M E2 for 3 and 6 days. Cell growth was determined in MTT assays. The increase in cell growth rates was calculated by subtracting from and dividing OD_{595} values with OD_{595} of control cells before treatment (time zero). **(C),** Control MCF-7 and Fe65 OE clone (2-5) were estrogen starved for 72 h and treated with 10^{-7} M E2 for 0, 3 and 6 days. E2 treatment was started at different times and cell growth of all treated cells was determined in MTT assays at the same time. For example, for 0 day treatment, the cells were treated with EtOH for 6 days. The increase in cell growth rates was calculated by subtracting from and dividing OD_{595} values with the value of control cells before E2 treatment (day zero). **(D),** Control MCF-7 and Fe65 OE clone (2-5) were estrogen starved for 72 h and treated with either 10^{-7} M E2 or 10^{-7} M E2 plus 1 μM TAM for 3 days. Cell growth was determined in MTT assays. Percentages of TAM inhibition were calculated by subtracting from E2 induced increase in growth rate (over day zero) with that induced by TAM, followed by division with E2 induced increase in growth rate. For all growth analyses, each data point represents 18 samples analyzed parallel. Each experiment was repeated three times. P-values were calculated by the Student's *t* test. The error bars in all growth analyses represent the standard deviation (SD). **(E),** A model describing the role of Fe65 in estrogen actions in neurons and breast cancer cells. See text for details.

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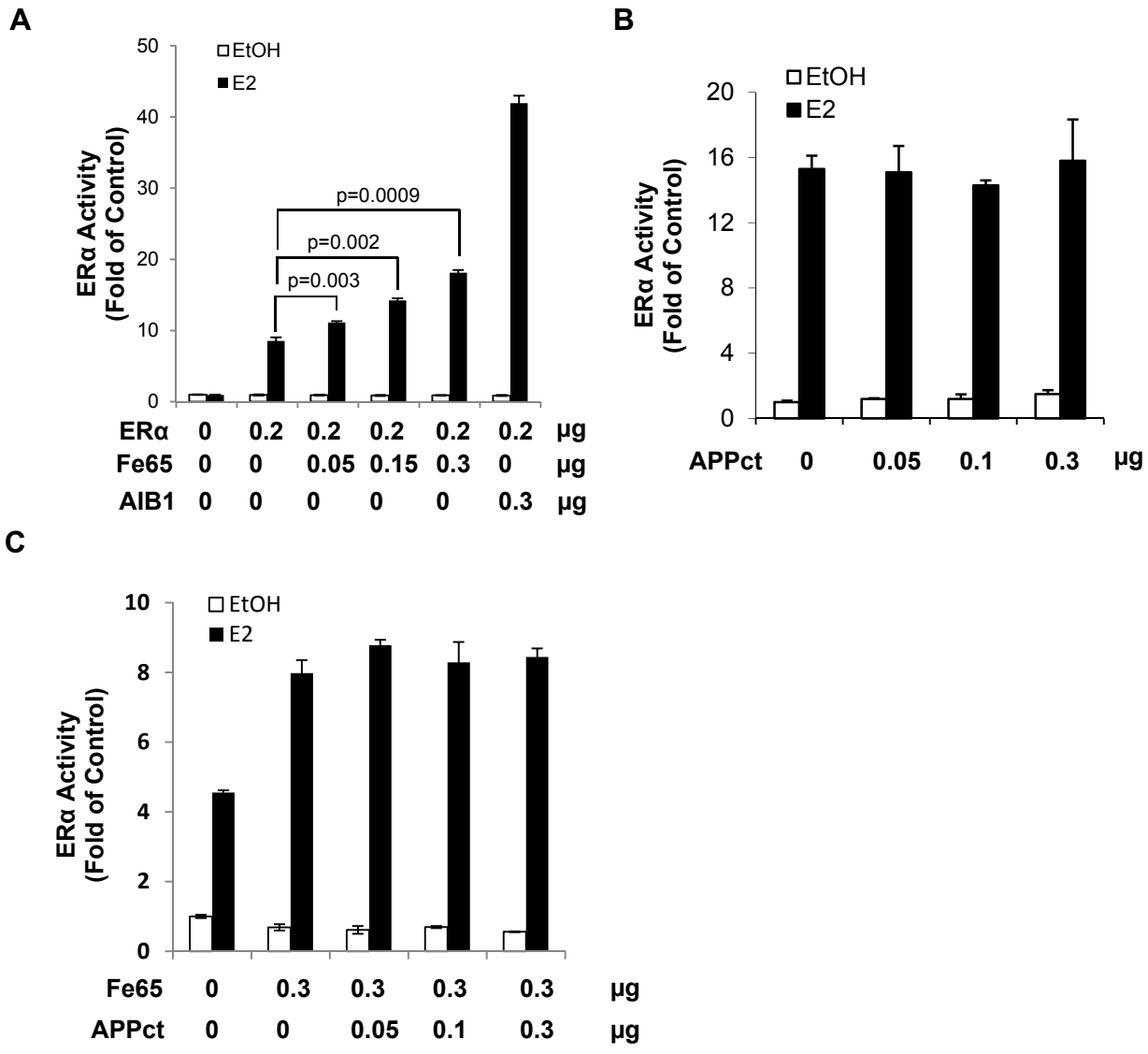
Fig 1.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Fe65 0 0 0.05 0.15 0.3 0 µg
AIB1 0 0 0 0 0 0.3 µg
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APPct 0 0.05 0.1 0.3 µg
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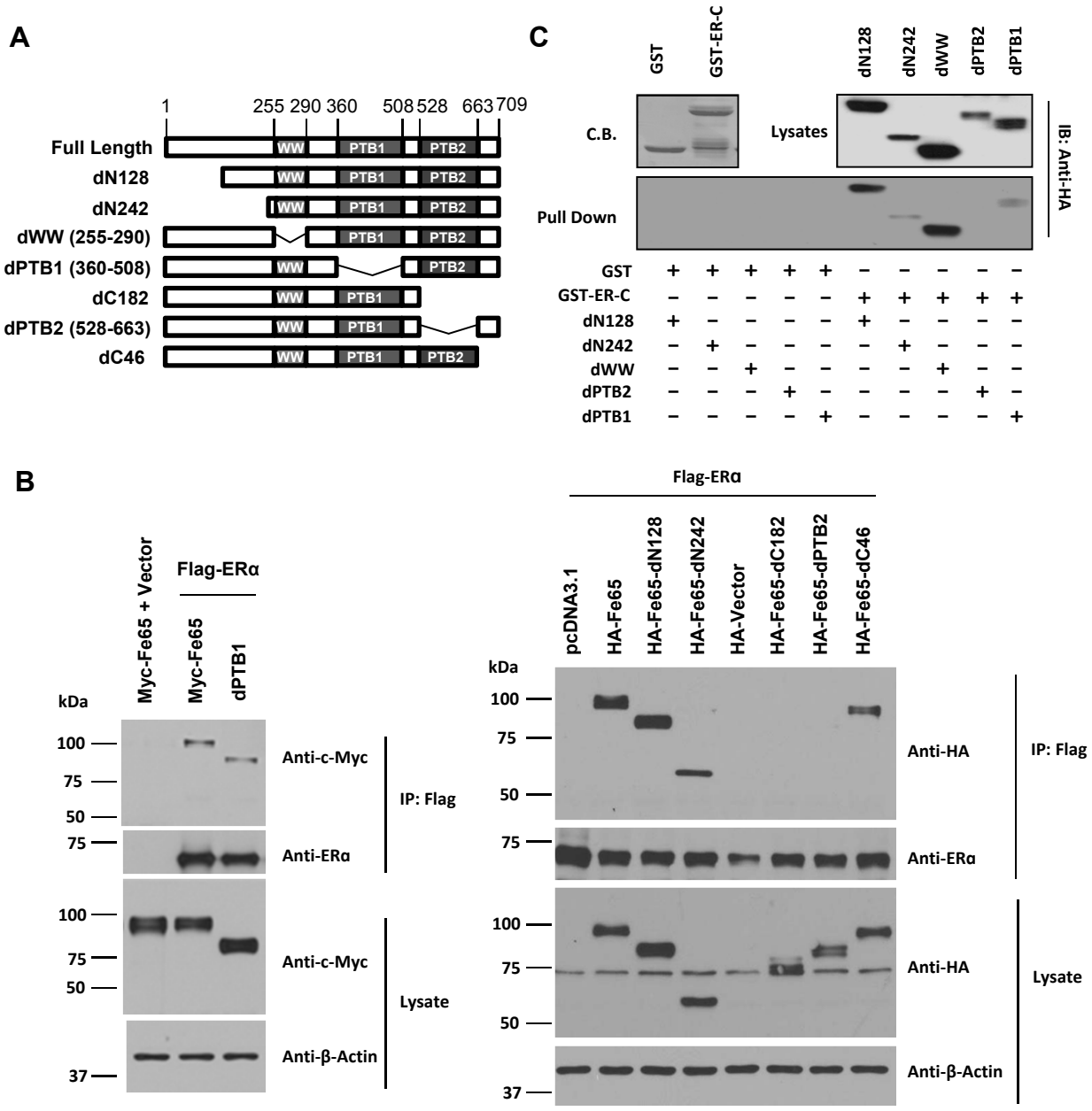
Fig 2.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WW
PTB1
PTB2
C
GST
GST-ER-C
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C.B.
Lysates
IB: Anti-HA
Pull Down
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GST-ER-C
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Flag-ERα
Myc-Fe65 + Vector
Myc-Fe65
dPTB1
kDa
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50
75
100
75
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Anti-c-Myc
Anti-β-Actin
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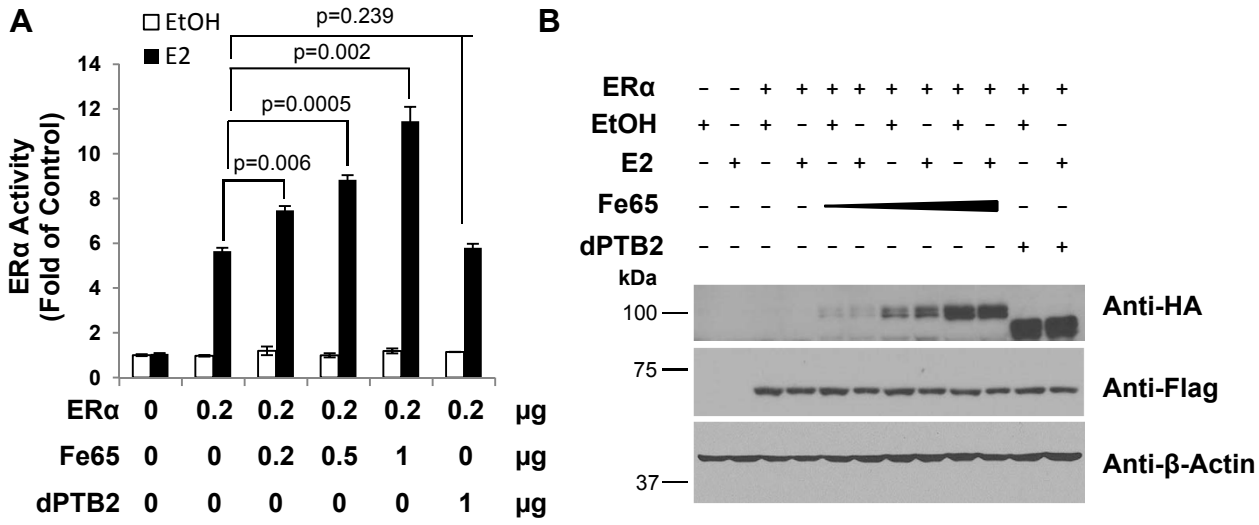
Lysate

Fig 3.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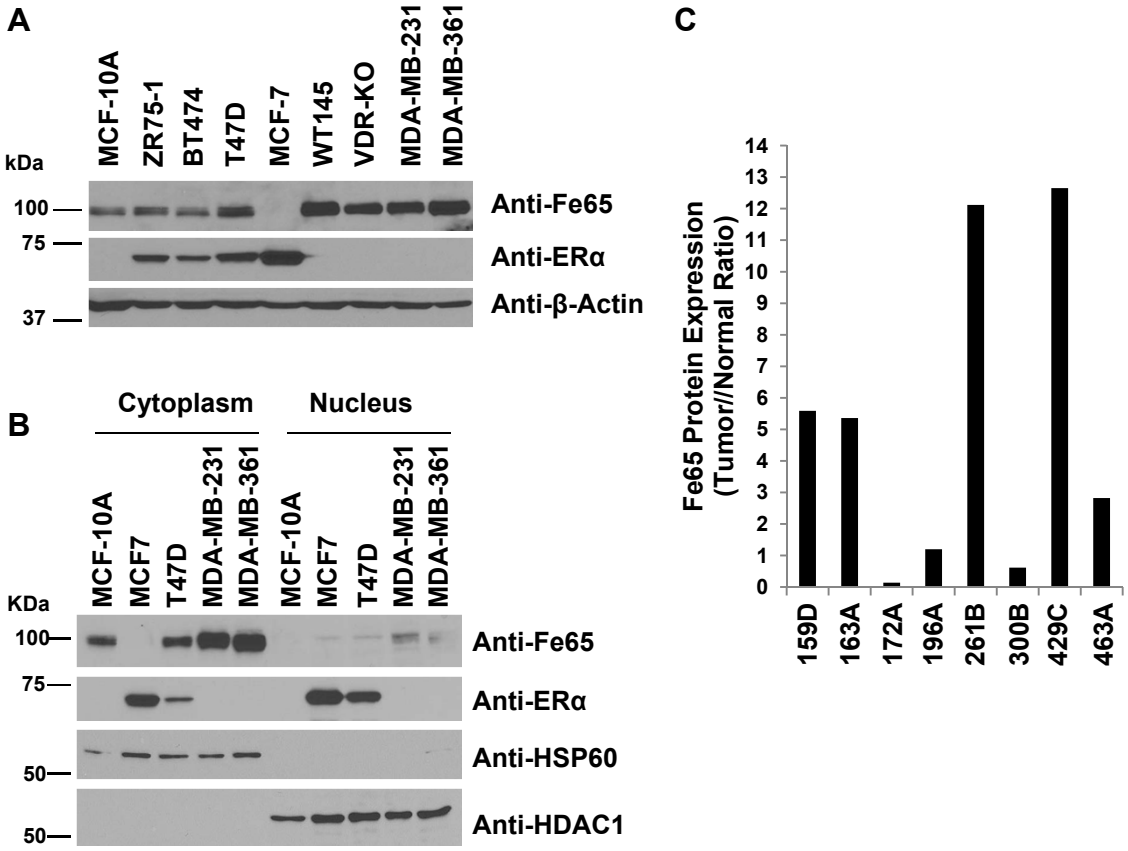
Anti-β-Actin

Fig 4.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Anti-ERα
Anti-β-Actin
B
Cytoplasm
Nucleus
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MCF7
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MCF7
T47D
MDA-MB-231
MDA-MB-361
KDa
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50
50
Anti-Fe65
Anti-ERα
Anti-HSP60
Anti-HDAC1
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2
3
4
5
6
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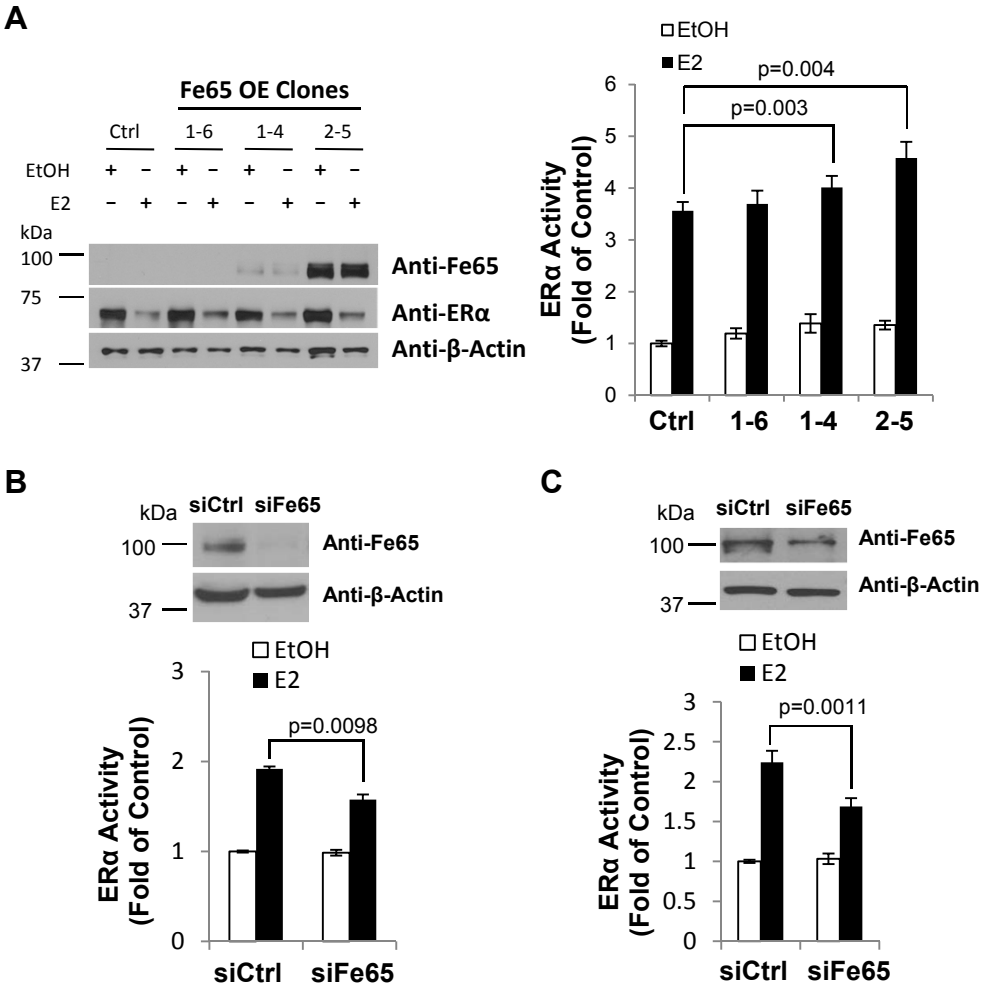
Fig 5.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1-4
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siFe65
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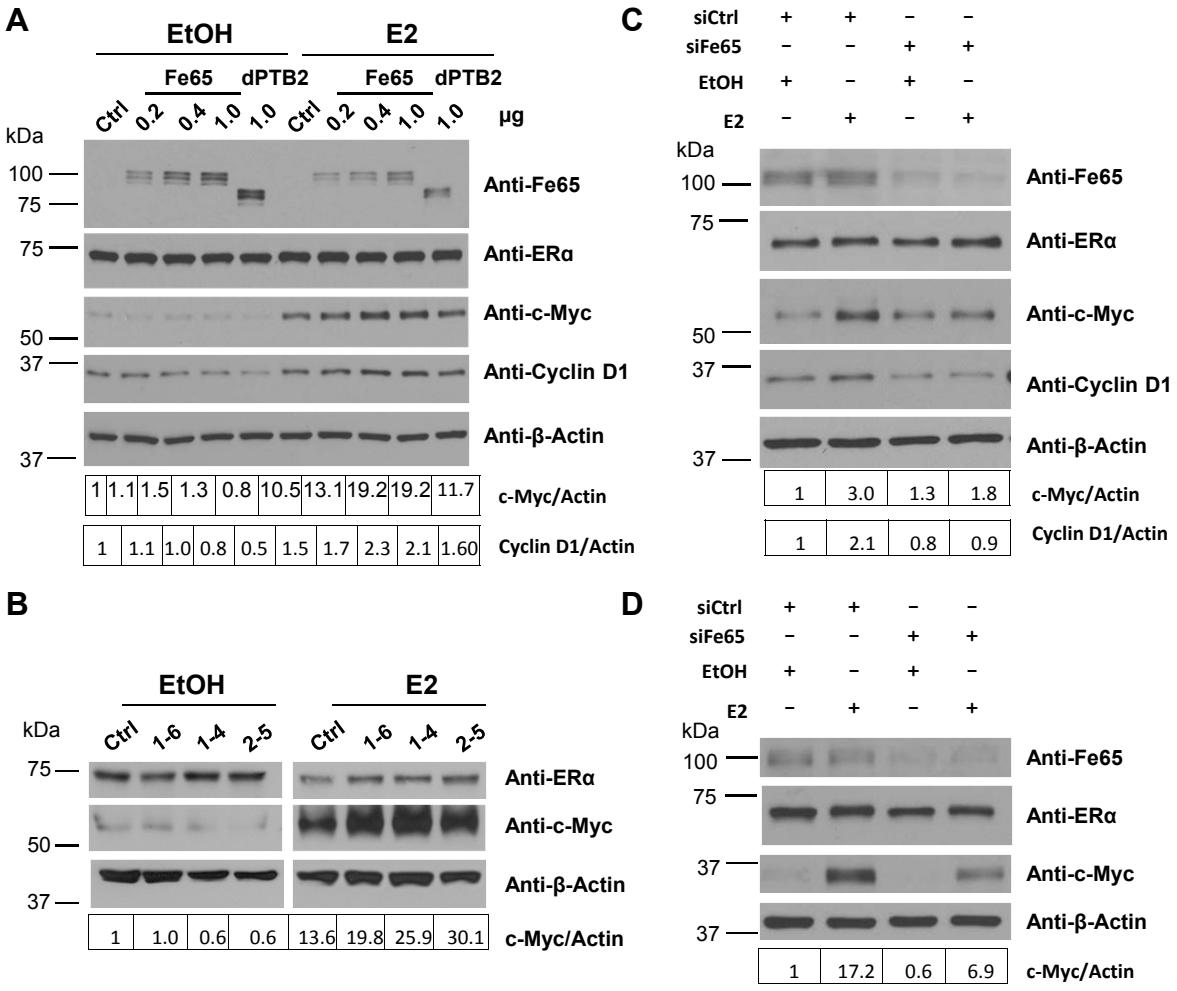
siFe65

Fig 6.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Anti-ERα
Anti-c-Myc
Anti-Cyclin D1
Anti-β-Actin
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c-Myc/Actin
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Cyclin D1/Actin
B
EtOH
E2
Ctrl
1-6
1-4
2-5
kDa
75
50
37
Anti-ERα
Anti-c-Myc
Anti-β-Actin
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c-Myc/Actin
C
siCtrl
siFe65
EtOH
E2
kDa
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Anti-Fe65
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Anti-c-Myc
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Anti-β-Actin
1 3.0 1.3 1.8
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Cyclin D1/Actin
D
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siFe65
EtOH
E2
kDa
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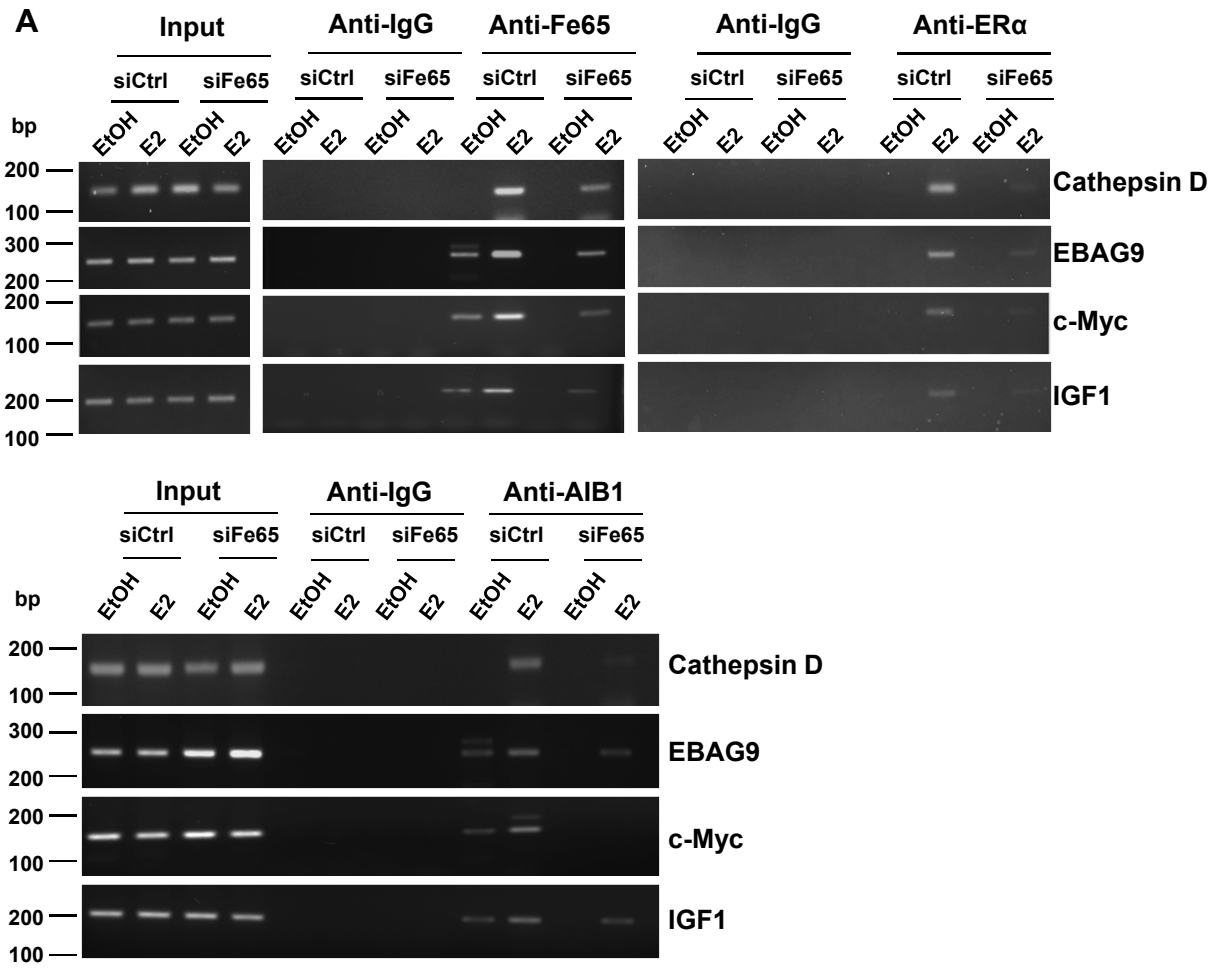
c-Myc/Actin

Fig 7.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Anti-IgG
Anti-AIB1

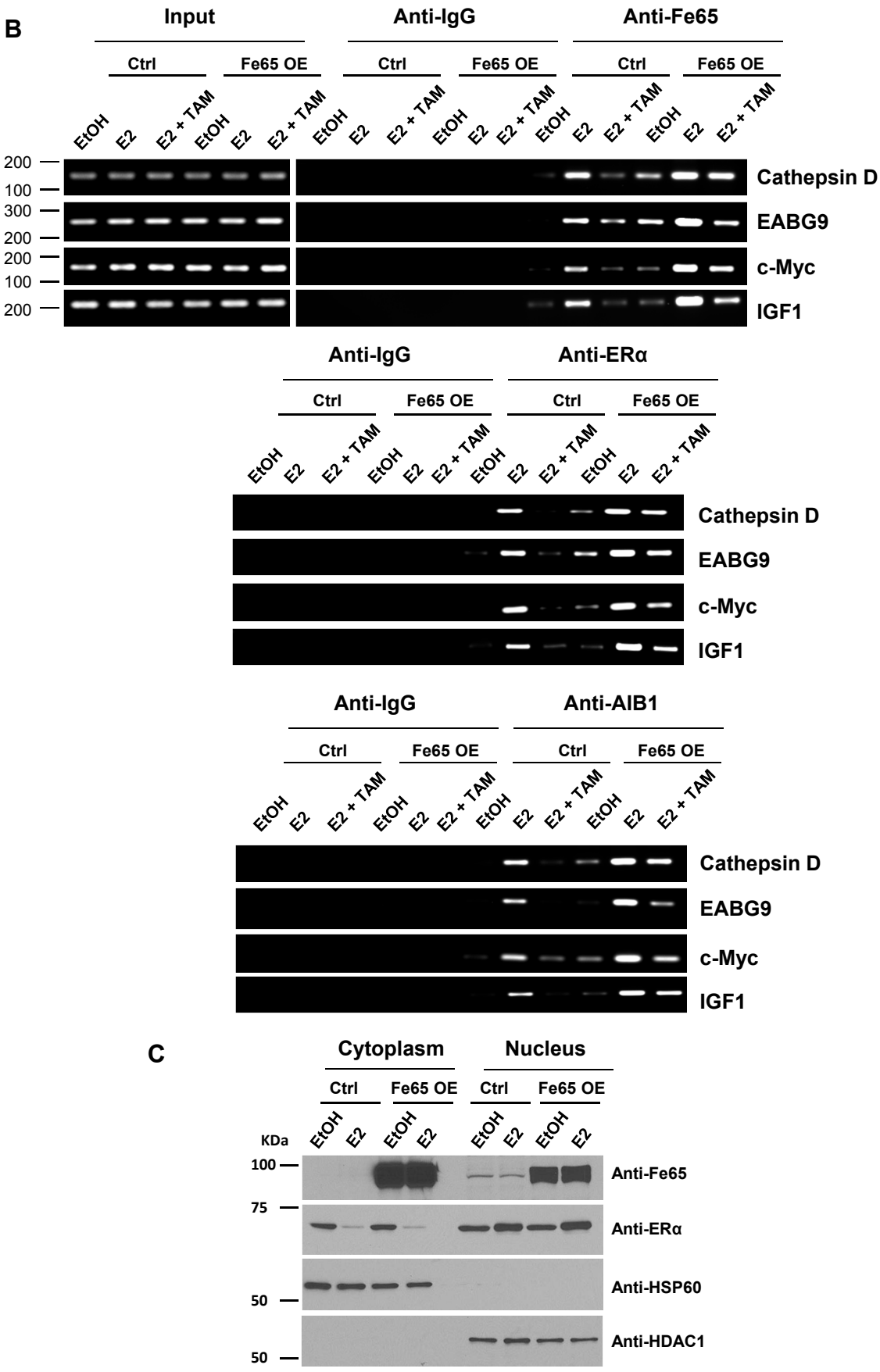
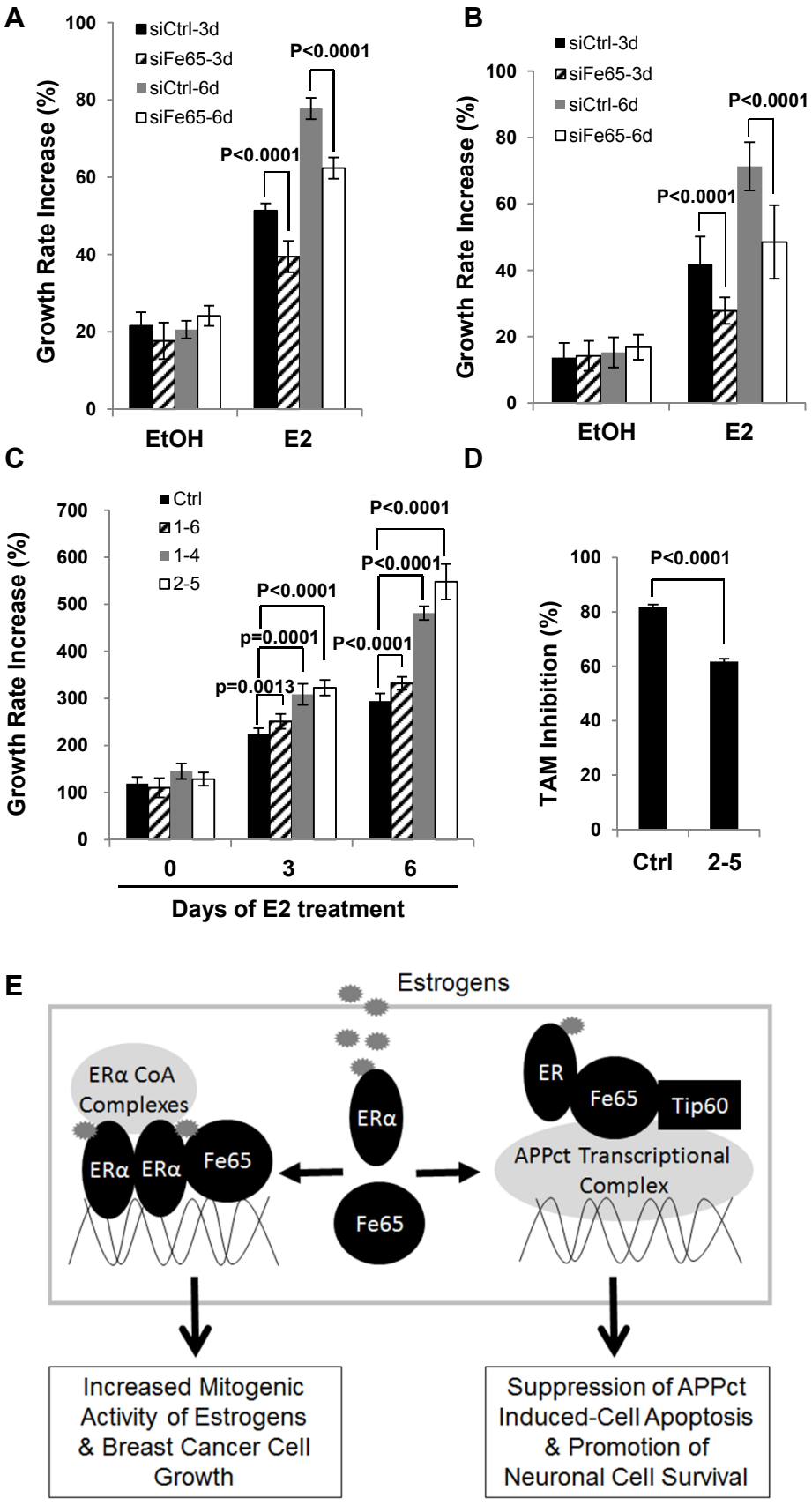

B
Input
Anti-IgG
Anti-Fe65
Ctrl
Fe65 OE
EtOH
E2
E2 + TAM
200
100
300
200
200
100
200
Cathepsin D
EABG9
c-Myc
IGF1
Anti-IgG
Anti-ERα
Anti-AIB1
C
Cytoplasm
Nucleus
KDa
100
75
50
50
Anti-Fe65
Anti-ERα
Anti-HSP60
Anti-HDAC1

Fig 8.


A
siCtrl-3d
siFe65-3d
siCtrl-6d
siFe65-6d
Growth Rate Increase (%)
P<0.0001
P<0.0001
EtOH
E2
B
siCtrl-3d
siFe65-3d
siCtrl-6d
siFe65-6d
Growth Rate Increase (%)
P<0.0001
P<0.0001
EtOH
E2
C
Ctrl
1-6
1-4
2-5
Growth Rate Increase (%)
P<0.0001
P<0.0001
P<0.0001
p=0.0001
P<0.0001
p=0.0013
0
3
6
Days of E2 treatment
D
TAM Inhibition (%)
P<0.0001
Ctrl
2-5
E
Estrogens
ERα CoA Complexes
ERα
ERα
Fe65
ERα
Fe65
ER
Fe65
Tip60
APPct Transcriptional Complex
Increased Mitogenic Activity of Estrogens & Breast Cancer Cell Growth
Suppression of APPct Induced-Cell Apoptosis & Promotion of Neuronal Cell Survival